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Salinity and Proteolytic Enzymes in Germinating Seeds of × *Haynaldoticum sardoum* (*Poaceae*)

By

Marina DEL ZOPPO*), Luciano GALLESCHI*), Antonino ONNIS**) and Franco SAVIOZZI*)

With 3 Figures

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Summary

DEL ZOPPO M., GALLESCHI L., ONNIS A. & SAVIOZZI F. 1998. Salinity and proteolytic enzymes in germinating seeds of × *Haynaldoticum sardoum (Poaceae)*. – Phyton (Horn, Austria) 38 (2): 281–291, with 3 figures. – English with German summary.

The effect of salinity on germination, early seedling growth, and storage protein mobilization of × *Haynaldoticum sardoum* MELETTI & ONNIS seeds was investigated. Increased salinity affected more adversely the early germination of the Culmo Pieno than Culmo Vuoto line seeds – the higher the temperature the greater the effect. The reduction in coleoptile and root growth appeared to be more sensitive to increased salinity than germination; minor differences between Culmo Pieno and Culmo Vuoto seedlings were found. The activity of some proteolytic enzymes involved in the mobilization of the endospermatic storage proteins was estimated: high salinity generally reduced the proteolytic activity. The inhibition of cysteine proteinase (EC 3.4.22) activity was particularly interesting since this enzyme was involved in the initial and further degradation of gluten, and appeared to play a key role in that process. The consequence of this inhibition could lead to the impaired capacity of the seed to mobilize storage proteins, and hence deprive young seedlings of nutrients prior to the development of the autotrophic capacity.

*) M. DEL ZOPPO, L. GALLESCHI, F. SAVIOZZI, Dipartimento di Scienze Botaniche, University of Pisa, Via L. Ghini 5, 56126-Pisa, Italy

**) A. ONNIS, Dipartimento di Agronomia e Gestione dell'Agroecosistema, University of Pisa, Via S. Michele degli Scalzi 4, 56124-Pisa, Italy.

Zusammenfassung

DEL ZOPPO M., GALLESCHI L., ONNIS A. & SAVIOZZI F. 1998. Salinität und proteolytische Enzyme in keimenden Samen von × *Haynaldoticum sardoum (Poaceae)*. – Phyton (Horn, Austria) 38 (2): 281–291, mit 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Es wurde der Einfluß von Salinität auf die Keimung, das frühe Sämlingswachstum und die Mobilisierung von Reserveproteinen bei Samen von × *Haynaldoticum sardoum* MELETTI & ONNIS untersucht. Steigende Salinität beeinflußte die frühe Keimung von Samen der Sorte Culmo Pieno nachteiliger als die der Sorte Culmo Vuoto – je höher die Temperatur, je größer die Wirkung. Die Reduktion im Wachstum der Koleoptilen und Wurzeln schien empfindlicher gegenüber steigender Salinität zu sein als die Keimung; geringere Unterschiede zwischen Culmo Pieno- und Culmo Vuoto-Sämlingen traten auf. Auch wurde die Aktivität einiger proteolytischer Enzyme, welche in die Mobilisierung der Reserveproteine des Endosperms eingebunden sind, untersucht: Hohe Salinität reduzierte ganz allgemein die proteolytische Aktivität. Die Beeinträchtigung der Aktivität der Cystein-Proteinase (EC 3.4.22) war besonders interessant, da dieses Enzym in den Beginn und den weiteren Abbau von Gluten eingebaut ist und eine Schlüsselrolle in diesem Prozeß zu spielen scheint. Diese Inhibition könnte konsequenterweise die Sämlinge beeinträchtigen, Reserveproteine zu mobilisieren. Dadurch werden jungen Sämlingen vor der Entwicklung ihrer autotrophen Kapazität Nährstoffe vorenthalten.

Introduction

Saline areas are mostly found in the arid and semiarid regions of the tropics and sub-tropics. In these areas, soil salinity has become an increasingly serious and costly problem for agriculture. Intense soil irrigation worsens the situation (FLOWERS & al. 1977), since the salts that have dissolved in water are left behind in the surface layers of the soil, as the water evaporates or is absorbed by plants. The surface layers thus become progressively more saline due to this secondary salinization process. The salinization of agricultural lands has serious consequences, as much of the land must ultimately be withdrawn from production. Unfortunately, most economically important crop species are very sensitive to saline soil conditions. This has stimulated studies to improve salt tolerance in traditional crops, and to identify potential new crop plants from wild species that can tolerate saline conditions.

The germination of non-dormant seeds is mainly influenced by the availability of water in the soil. The presence of excessive salinity, which decreases the water potential of the soil solution, restricts water uptake and inhibits germination. After germination, seedling growth commences and the presence of excessive soil salinity, by decreasing water uptake, can delay seedling emergence. During this development period the seedling is still not autotrophic and hence is dependent on the nutrients provided by the hydrolysis of seed storage reserves. Since the mobilization of these reserves represents one of the most critical post-germinative events in

growth and seedling development, it is important to understand if and how it is influenced by salinity. The present investigation was undertaken on seeds of ×*Haynaldoticum sardoum* MELETTI & ONNIS, an hexaploid allopolyploid hybrid probably originating from *Haynaldia villosa* (L.) Schur. and *Triticum durum* Desf. (MELETTI & ONNIS 1975, STEFANI & al. 1987). ×*H. sardoum* grows exclusively among crops of *Triticum durum* in Sardinia, Sicily, central and southern Italy. This wild wheat is characterized by two lines, one with a solid stem (Culmo Pieno) and the other with a hollow stem (Culmo vuoto; MELETTI & ONNIS 1975). CP wheat has spring habit, while CV wheat has winter habit. A preliminary study has shown that the early germination of CP seeds is more susceptible than CV seeds to NaCl (CREMONINI & al. 1992).

The aim of this research was firstly to identify possible sources of salt-tolerance characteristics in ×*Haynaldoticum* which may be used to improve the salt tolerance of wheat through hybridization, and secondly to investigate whether salinity affects the mobilization process of storage proteins. Seed germination, seedling growth, and changes in proteinase, carboxypeptidase, and aminopeptidase activities were examined.

Abbreviations: BZ-Asn-pNA, benzoyl-asparaginyl-p-nitroanilide; CBZ-Phe-Ala, carbobenzoxy-phenylalanyl-alanine; CP, Culmo Pieno; CV, Culmo Vuoto; Leu-pNA, leucine p-nitroanilide.

Materials and Methods

Plant material

Seeds of ×*Haynaldoticum sardoum* of the CP and CV lines were utilized. They were collected in 1995 in S. Piero a Grado (Pisa, Italy) and stored at 10° C in closed glass jars from harvest until used. The seeds were surface-sterilized in 1% NaOCl for 20 min, washed in distilled water, and allowed to germinate in Petri dishes (fifteen seeds in three dishes for each line) on Whatman number 2 filter paper imbibed with 4 ml of distilled water or with the same volume of NaCl solutions (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 M). Germination and seedling growth were performed at 23 and 30° C in the dark. Seeds with a radicle protrusion of up to 5 mm were counted as having germinated. Seedling growth was assayed after 96 h by measuring elongation of coleoptile and roots.

Extraction of proteolytic enzymes

The extraction of proteolytic enzymes was performed on 1500 seeds for each line. These seeds were surface-sterilized with 1% NaOCl, washed in distilled water, and allowed to germinate in seed trays for 24 h at 23° C in the dark. Seedlings were transferred to identical seed trays containing water, 0.05 and 0.2 M NaCl. The imbibition continued until the third day. The endosperms were then isolated, frozen, and lyophilized. The lyophilized endosperms were ground and the meal (5 g for each extraction) was resuspended with 0.2 M potassium phosphate buffer (pH 6) containing 2-mercaptoethanol, at a ratio 1:3 (w/v), and extracted for 1 h at 4° C. The extract was centrifuged at 40 000 g and 4° C for 10 min. The supernatant was brought to 80% saturation with solid ammonium sulfate, and centrifuged as above. The precipitate

was redissolved in 3 ml of 0.02 M potassium phosphate buffer (pH 6), containing 2.5 mM 2–mercaptoethanol and dialysed overnight at 4° C against 1 l of the same buffer. The dialysate was cleared by centrifugation at 40 000 g for 10 min, and the supernatant was utilized as an enzymic source.

Enzyme assays

The proteinase activity was assayed using gliadin (BIGIARINI & al. 1995) and BZ-Asn-pNA (BOTTARI & al. 1996b) as substrates. One unit of gliadin hydrolysing activity is the amount of enzyme that liberates 1 nmol α-amino nitrogen per minute at 30° C. One unit of Asn-endopeptidase activity is defined as the amount of enzyme that yields 1 nmol of pNA per minute at 30° C. The aminopeptidase activity was assayed with Leu-pNA following GALLESCHI & al. 1988. One unit of aminopeptidase activity is defined as the amount of enzyme that liberates 1 nmol of pNA per minute at 30° C. The carboxypeptidase activity was assayed with CBZ-Phe-Ala, determined as previously described (GALLESCHI & CAPOCCHI 1986). One unit of carboxypeptidase activity is defined as the amount of enzyme that releases 1 nmol of alanine per minute at 30° C. The protein content was estimated following LOWRY & al. 1951, using bovine serum albumin as the standard.

The results were analysed by a one-way analysis of variance, using the CoStat statistical package (CoHort 2 Software, 1986, 1990).

Results

Table 1 shows the effects of salinity on germination of CP and CV seeds. At 23° C and after 24 h, the germination capacity was higher in CV than in CP seeds for all concentrations examined up to 0.3 M; a similar behaviour was observed after 48 h. After 72 and 96 h the maximal germination capacity was reached. Minor differences were found between the seeds of the two lines, i.e. the slow germination (7%) in 0.6 M NaCl of the CV seeds compared with the absence of germination of the CP seeds. When the temperature was increased, the germination capacity of the CP seeds was lower than the CV seeds.

Table 2 shows the effect of salinity on the growth of CP and CV seedlings. At 23° C, the increased salinity progressively reduced the growth of coleoptile and roots in CP and CV seedlings. When NaCl concentration was equal to or higher than 0.3 M growth stopped in both types of seedlings. At 30° C and in the presence of low salinity, the growth of coleoptile and roots increased, while, when the salt concentration was increased, the growth was severely reduced.

Since these results showed a reduction in seedling growth up to 0.2 M NaCl for both temperatures used, the study on the mobilization of storage proteins was performed at 23° C in the presence of 0.05 and 0.2 M NaCl. Furthermore, endosperms from 4-day-old seedlings were used as the starting material for the enzyme assays, since our previous studies (GALLESCHI & PELLEGRINI 1989, BOTTARI & al. 1996a, b) had shown that proteolytic activities were most prominent during this period.

Table 1

Germination capacity (%) of ×*Haynaldoticum sardoum* seeds at different temperatures and NaCl concentrations. Values are means ± SE of three replicates of 15 seeds each. Values in the same column followed by different letters are significantly different ($P < 0.01$). CP, solid stem; CV, hollow stem.

		23°				30°			
	NaCl (M)	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
CP	**0**	76±4.4 a	89±5.9 ab	100 a	100 a	71±4.4 a	87±0.0 a	100 a	100 a
	0.05	62±2.2 b	83±1.9 b	95±2.2 b	100 a	55±2.2 b	71±2.2 b	100 a	100 a
	0.1	47±6.7 c	91±2.2 a	95±2.2 b	100 a	40±0.0 c	87±3.8 a	100 a	100 a
	0.2	29±4.4 d	73±3.8 c	89±2.2 c	89±2.2 b	15±2.2 d	55±2.2 c	67±0.0 b	67±0.0 b
	0.3	2±0.2 e	24±2.2 d	89±2.2 c	89±2.2 b	0 e	15±2.2 d	31±2.2 c	31±2.2 c
	0.4	0 f	4±1.2 e	24±2.2 d	51±2.2 c	0 e	0 e	0 d	0 d
	0.6	0 f	0 f	0 f	0 e	0 e	0 e	0 d	0 d
CV	**0**	95±2.2 a	98±2.2 a	100 a	100 a	91±4.4 a	100 a	100 a	100 a
	0.05	87±3.8 b	96±4.4 ab	100 a	100 a	91±2.2 a	98±2.2 ab	100 a	100 a
	0.1	87±3.8 b	96±4.4 ab	100 a	100 a	91±4.4 a	95±2.2 b	100 a	100 a
	0.2	60±3.9 c	93±3.8 b	95±2.2 b	95±2.2 b	67±3.8 b	82±4.5 c	100 a	100 a
	0.3	18±4.5 d	69±2.2 c	84±4.4 c	87±3.8 c	9±2.2 c	64±2.2 d	91±2.2 b	98±2.2 a
	0.4	0 e	51±5.9 d	67±0.0 d	73±3.8 d	0 d	0 e	64±5.9 c	64±5.9 b
	0.6	0 e	0 e	7±3.9 e	7±3.9 e	0 d	0 e	0 d	0 c

Table 2

Root and coleoptile growth (cm) after 96 h of × *Haynaldoticum sardoum* seedlings at different temperatures and NaCl concentrations. Values are means ± SE of three replicates of 15 seedlings each. Values in the same column followed by different letters are significantly different ($P < 0.01$). The absence of values is due to no germination or to no measurable growth. CP, solid stem; CV, hollow stem.

		23°		30°	
	NaCl (M)	Roots	Coleoptile	Roots	Coleoptile
CP	**0**	7.9±0.20 a	5.2±0.16 a	8.3±0.15 a	5.5±0.14 a
	0.05	5.3±0.11 b	3.1±0.12 b	6.5±0.13 b	4.2±0.14 b
	0.1	2.9±0.09 c	1.9±0.09 c	3.9±0.10 c	2.2±0.10 c
	0.2	1.1±0.07 d	0.5±0.06 d	0.6±0.12 d	0.4±0.07 d
	0.3	–	–	–	–
	0.4	–	–	–	–
	0.6	–	–	–	–
CV	**0**	7.6±0.18 a	5.2±0.18 a	8.3±0.15 a	6.3±0.14 a
	0.05	5.5±0.10 b	3.2±0.12 b	6.2±0.14 b	4.4±0.12 b
	0.1	3.6±0.10 c	2.5±0.10 c	4.1±0.11 c	2.8±0.12 c
	0.2	1.0±0.07 d	0.5±0.06 d	0.4±0.09 d	0.2±0.06 d
	0.3	–	–	–	–
	0.4	–	–	–	–
	0.6	–	–	–	–

Figure 1 shows the variations in dry weight and total proteins of endosperms from CP (A) and CV (B) seeds of × *Haynaldoticum sardoum* after 24 h of germination and following transfer to NaCl solutions. When the salt concentration was increased, the endosperm dry weight remained similar to that of the control that had germinated for 24 h. The reduction in total protein was moderately affected by the presence of a low salt concentration, and was strongly reduced by high salinity.

The gliadin degrading activity from endosperms of the CP seeds, which was just detectable after 24 h of germination in water (Fig. 2A), increased similarly in the presence of water and 0.05 M NaCl after 96 h of germination. On the contrary, a high salt concentration almost completely prevented the increase in activity. A similar pattern in the gliadin degrading activity was observed in the CV seeds, although the decrease in activity was already evident in the presence of 0.05 M NaCl and more pronounced in the presence of 0.2 M NaCl.

The Asn-endopeptidase activity increased after germination similarly in water and in the presence of a low salt concentration for the CP seeds, while a further increase was found for the CV seeds (Fig. 2B). High salinity almost completely prevented the increase in the activity in CP and CV seeds.

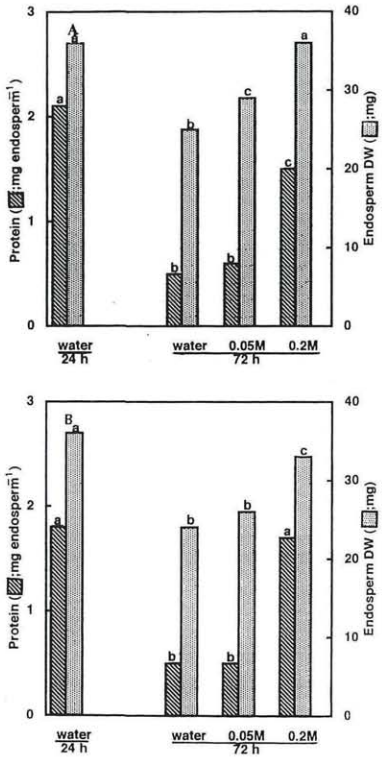

Fig. 1. Dry weight and protein content in CP (A) and CV (B) endosperms from seeds of ×*Haynaldoticum sardoum* imbibed 24 h in water and then transferred to water and to 2 NaCl concentrations. Values are means of three replicates of 15 seeds each.

The aminopeptidase activity increased similarly after the CP seed germination in the presence of water and 0.05 M NaCl (Fig. 3A); the CV seeds showed a higher activity in 0.05 M NaCl than in water. A high salt concentration completely prevented the increase in activity for both seeds.

Finally, the carboxypeptidase activity increased after the germination of the CV seeds in water, and even more in the presence of 0.05 M NaCl (Fig. 3B). The CP seeds showed a moderate decrease in activity in the presence of 0.05 M NaCl. A high salt concentration completely prevented the increase in activity.

Discussion

Studies of the effect of salinity on the mobilization of seed reserves are scarce and incomplete. SHEORAN & GARG 1978 studied the influence of salinity on RNAase, DNAase and protease activities during the germina-

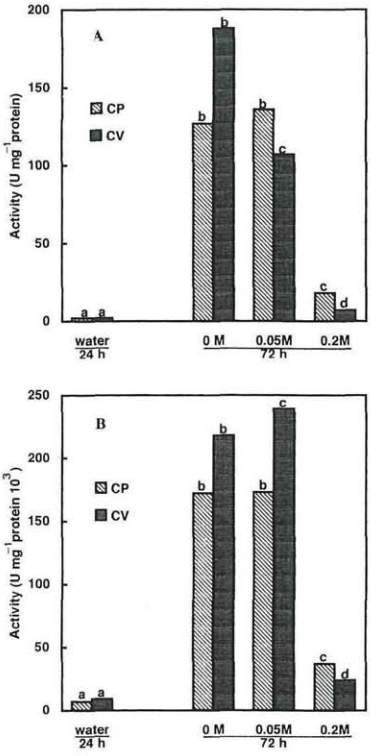

Fig. 2. Proteinase activities hydrolysing gliadin (A) and BZ-Asn-pNA (B) in CP and CV endosperms from seeds of × *Haynaldoticum sardoum* imbibed 24 h in water and then transferred to water and to 2 NaCl concentrations. Values are means of 8 replicates.

tion of mung bean seeds. They found that treatment with a high NaCl concentration reduced the protease activity in cotyledons and the embryo axis during early seed germination. However, they assayed the protease activity only with an exogenous substrate, and did not classify the enzyme in order to prove its involvement in the mobilization of storage proteins. Dubey & Rani 1990 studied the effect of salinity in rice seedlings with different salt tolerances. They examined the proteinase and exopeptidase activities extracted from roots and shoots, and found that salinity leads to a marked increase in the activity of proteolytic enzymes, particularly in salt tolerant seedlings. They showed that an increased rate of proteolysis under salt stress conditions was associated with higher activities of proteinase, aminopeptidase and carboxypeptidase in susceptible rice cultivars, whereas was associated with higher activities of proteinase and aminopeptidase in salt tolerant cultivars. The authors hypothesized that the

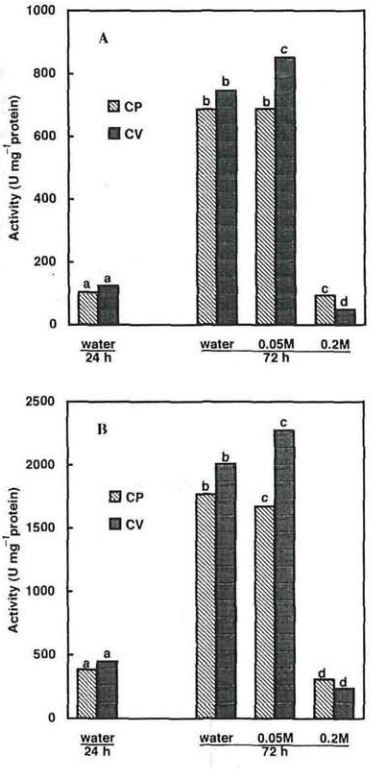

Fig. 3. Exopeptidase activities hydrolysing Leu-pNA (A) and CBZ-Phe-Ala (B) in CP and CV endosperms from seeds of ×*Haynaldoticum sardoum* imbibed 24 h in water and then transferred to water and to 2 NaCl concentrations. Values are means of 8 replicates.

increased levels of proteinase and aminopeptidase could be involved in conferring salt tolerance to rice seedlings.

Since the hydrolysis of storage proteins to soluble products supports seedling growth prior to the development of autotrophy, an inhibition of this degradative process might prevent normal seedling development. It is known that cereal storage proteins are degraded during early seedling growth by endo- and exopeptidases (BEWLEY & BLACK 1994). Cysteine proteinases, which appear in the endosperm of cereal seeds during the germination process, seem to play a key role (BIGIARINI & al. 1995). Such an enzyme has been purified and characterized from endosperms of germinating wheat: it initiates the degradation of gliadin and participates in its further hydrolysis to small peptides (BOTTARI & al. 1996a). It is also able to degrade glutelins, extracted from the same seeds, to small soluble products (GALLESCHI, unpublished data). A similar activity has been detected in

endosperms from germinating seeds of ×*Haynaldoticum sardoum* (GALLESCHI & al. 1989), where, presumably, the enzyme carries out the same role. In the present work Asn-endopeptidase activity has been detected for the first time in endosperms from germinating ×*H. sardoum* seeds, although such an enzyme had been already found in germinating wheat (BOTTARI & al. 1996b). Preliminary data, based on the tissue localization, suggest that the wheat Asn-endopeptidase might be involved in the degradation of storage proteins deposited in the aleurone layer and not in that of the storage proteins present in the starchy endosperm (BOTTARI & al. 1996b).

Endosperms from germinating seeds of ×*Haynaldoticum sardoum* also contain well characterized carboxypeptidase (GALLESCHI & CAPOCCHI 1986) and aminopeptidase (GALLESCHI & PELLEGRINI 1989) activities. Both activities appear to be involved in the hydrolysis of products derived from the action of endopeptidases.

The presence of high salinity appears to inhibit the process of storage protein mobilization in ×*Haynaldoticum sardoum* seeds. In fact, both proteinase and exopeptidase activities are several times lower than the controls in water. These results contrast with the data found by DUBEY & RANI 1990 and with their hypothesis attributing key roles to proteinase and aminopeptidase in conferring salt tolerance to rice seedlings. The discrepancy might be due to differences in the species and in the tissues studied, that is roots and shoots for rice and endosperms for ×*H. sardoum*, but also to the use of an exogenous protein (casein) to assay the proteinase activity. In fact the risk is to assay a proteolytic enzyme irrespective of its function (SHUTOV & VAINTRAUB 1987). In addition the inhibition of the cysteine proteinase activity appears to be particularly interesting, since it represents the major hydrolytic activity present in the endosperm of wheat (BOTTARI & al. 1996a) and ×*H. sardoum*. Since the enzyme is able to degrade gluten to small soluble peptides, its inhibition impairs the mobilization process in the endosperm. Moreover, the very low exopeptidase activities would worsen the situation by impeding the supply of amino acids to the growing seedling. The cumulative result would be a much reduced embryo growth.

In conclusion, although ×*H. sardoum* shows a competitive behaviour among crops and a certain tolerance to low salt concentrations, above all in the CV seeds during early germination, seedling growth has been found to be susceptible to high salinity. This salinity acts at a molecular level by inhibiting the mobilization process of storage proteins.

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